CCLXII. AEROBIC BREAKDOWN OF GLUCOSE BY *BACT. SUBOXYDANS*.

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BACT. SUBOXYDANS, an acetic acid organism isolated by Kluyver & de Leeuw [1924], is described by them as "characterized by its slight intensity of oxidation which leads to an accumulation of intermediary products". Normally, in aerated cultures grown in suitable liquid media, *Bact. suboxydans* converts glucose into gluconic acid and sometimes further into 5-ketogluconic acid. In this respect it differs considerably from related organisms such as *Bact. aceti*, *Bact. rancens* and *Bact. xylinum*, which are capable of oxidizing both glucose and gluconic acid to carbon dioxide and water. In the work to be described it is shown that the property of restricted oxidation so characteristic of *Bact. suboxydans* in aerobic conditions can be changed into one of strong oxidative ability similar to that of the three organisms mentioned. Moreover, the two types of culture possessing these different oxidative properties are convertible one into the other by a simple modification of the medium on which the organism is grown.

The present investigation shows that two very different types of suspension are obtained when *Bact. suboxydans* is grown on maize wort agar or on maize wort agar to which 2% chalk has been added. Suspensions derived from the medium without chalk are dirty white in colour and are capable of oxidizing glucose to gluconic acid only. They give no oxygen uptake or evolution of carbon dioxide with gluconic acid. Suspensions from cultures grown on the medium containing chalk are pink and give a very much larger and more rapid oxygen uptake with glucose than the other type of suspension, as well as a considerable evolution of carbon dioxide. Moreover, with gluconic acid a rapid oxygen uptake and evolution of carbon dioxide are obtained. It is concluded from the experimental figures that with this type of suspension glucose is first oxidized to gluconic acid, which is then broken down with evolution of carbon dioxide equal to the volume of oxygen absorbed. Some of the glucose or gluconic acid is probably oxidized to a keto- or aldehydo-gluconic acid. It is of interest in connexion with the large increase in oxygen uptake resulting from growth of *Bact. suboxydans* on the medium containing chalk, that in a recent publication Cozic [1936] states that a considerable increase in the rates of oxygen absorption by suspensions of *Bact. rancens* and *Bact. pasteurianum* was observed when chalk was added to the growth medium. It should also be mentioned that Simon [1930] showed that *Bact. suboxydans*, together with *Bact. pasteurianum* and *Bact. ascendens*, can act anaerobically on glucose to give a true alcoholic fermentation, but he states that this does not occur in the presence of oxygen.

The striking difference in oxidative powers and of ability to break down the glucose molecule of the two types of suspensions implies that the enzymic system possessed by suspensions of *Bact. suboxydans* grown on maize wort agar containing chalk is profoundly different in function from that possessed by

suspensions derived from cultures grown on maize wort agar without chalk. It is very probable that the difference is due directly to the neutralization by the chalk of the acid formed by the organism during growth on the medium. Without chalk the strongly acid conditions which develop are probably unfavourable for the formation in the enzyme system of the organism of the specific active group responsible for the breakdown of the glucose or gluconic acid molecule. It is also possible that the pink pigment present in the more active suspension is partly responsible for the increased activity by acting as carrier between the activated substrate molecules and molecular oxygen, in much the same way as other intracellular pigments such as the well-known yellow enzyme and the blue pigment of *Bact. pyocyaneum*.

An attempt has been made to link this difference in properties with the intensity of phosphatase activity in the suspensions.

EXPERIMENTAL.

Bacterial suspensions and preparations. The culture of *Bact. suboxydans* used in these experiments was obtained from the National Collection of Type Cultures. The history of the different subcultures previous to use in the experiments described had varied considerably. For most of the time all cultures were on maize wort agar, but at some period they had been on beer agar, ordinary wort agar, yeast-glycerol liquid medium, bouillon-agar and maize wort agar with chalk. The media employed for growth in the experiments to be described consisted of (a) maize wort agar, (b) maize wort agar with 2% chalk, contained in flat 16-oz. bottles. In order to incorporate the chalk throughout the agar it was essential to shake the bottles just before solidification. A thin saline suspension of the organism grown on the same medium was used for inoculation and the bottles were incubated at 30° for 4 days. The growth was removed from the agar surface by adding sterile physiological saline and rubbing with a sterile glass rod. The washings thus obtained from the maize wort agar without chalk were very acid and, to remove this acidity it was necessary, after filtering through sterile glass wool to remove small lumps of agar, to centrifuge and wash three or four times before finally suspending in saline. The organisms from the chalk medium were much more difficult to centrifuge and gave a definitely pink suspension, in contrast to the dirty white of the other suspensions. The suspensions were aerated for 1 hour and kept at 0°. The dry weights of the suspensions from the maize wort agar without chalk were usually 10–15 mg./ml. and those from the chalk medium 20–25 mg./ml. Dilution within a wide range made no appreciable difference; e.g. a suspension from the chalk medium, with dry weight 23 mg./ml., gave almost identical values for oxygen uptake and evolution of carbon dioxide with glucose when diluted to three times its volume.

The air-dried preparations were made by centrifuging a portion of the suspensions, drying the sediment in a vacuum desiccator and grinding to a fine powder.

The acetone preparations were made from the suspensions as described by Müller [1931].

Oxygen absorption and evolution of carbon dioxide of suspensions of Bact. suboxydans with glucose and calcium gluconate. These were measured by means of the Barcroft differential manometer, the usual technique being followed [Dixon, 1934]. The volumes of the two flasks in each apparatus were so adjusted by means of glass beads that their difference was less than 100 μ l. The carbon dioxide output was measured by the direct method. The left flask of the manometer contained 1 ml. suspension and 2 ml. buffer, the right flask 1 ml. suspension, 1.7 ml. buffer and 0.3 ml. substrate contained in a Keilin tube during equilibration, after which it was mixed with the other contents. Either phosphate or phthalate buffers of pH 5.5 were used, preliminary experiments having shown that 5.5 is the optimum pH for oxygen absorption by *Bact. suboxydans* with glucose as substrate. 0.1 *M* glucose was used. Theoretically 0.3 ml. of 0.1 *M* glucose takes up 334 μ l. of oxygen at N.T.P. for each atom of oxygen absorbed by the glucose molecule. A temperature of 30° was used throughout.

Suspensions of Bact. suboxydans grown on maize wort agar (no chalk).

Fig. 1 shows an oxygen uptake-time curve typical of many which have been obtained at different times with glucose as substrate. The oxygen uptake was comparatively rapid at the beginning and slowly decreased until it ceased altogether at a value of 320–340 μ l. oxygen, i.e. at a value equivalent to the absorption of one atom of oxygen by each molecule of glucose represented by 0.3 ml.

Fig. 1. Oxygen uptake, with 0.3 ml. of 0.1 *M* glucose as substrate, of suspension of *Bact. suboxydans* from growth on maize wort agar without chalk. Theoretical O_2 uptake for complete oxidation to gluconic acid is 334 μ l. Temp. 30°. pH, 5.5.

substrate. No carbon dioxide was evolved. The increase in acidity of the right-hand flask containing suspension and glucose almost invariably amounted to 1.5 ml. of 0.02 *N* NaOH, which is equivalent to the gluconic acid obtainable from 0.3 ml. of 0.1 *M* glucose. The absorption of the equivalent of one atom of oxygen and a simultaneous appearance of an equivalent acidity points very strongly to the formation of gluconic acid. The only other possible position for a carboxyl group in the intact glucose molecule would be at the other end of the chain and if this were formed it would require the absorption of the equivalent of two atoms of oxygen.

There was no oxygen uptake or evolution of carbon dioxide with 0.05 *M* calcium gluconate at pH 5.5.

It was thought possible that the thorough washing which this type of suspension required to remove all acid might also have removed some active agent of the enzymic system. Accordingly a portion of a suspension was centrifuged, the supernatant liquid removed and the suspension made up again with some of the neutralized supernatant liquid of the first washing. The new suspension was examined for oxygen absorption and evolution of carbon dioxide in presence of glucose in the Barcroft differential manometer and was found to give similar results to the above. It was concluded that washing had had no de-activating effect.

Suspensions of Bact. suboxydans grown on maize wort agar containing 2% chalk.

Fig. 2 shows a typical example of an oxygen uptake-time and CO_2 output-time curve obtained with this type of suspension using 0.3 ml. of 0.1 *M* glucose as substrate. The initial uptake of oxygen is much more rapid than that obtained with the type of suspension derived from *Bact. suboxydans* grown without chalk. The rate of oxygen absorption slowly diminishes, especially after the equivalent of one atom of oxygen has been taken up. At first there is only very little, if any, evolution of carbon dioxide, but this rapidly increases. After a

Fig. 2.

Fig. 3.

Fig. 2. Oxygen uptake and CO_2 output, with 0.3 ml. 0.1 *M* glucose as substrate, of suspension of *Bact. suboxydans* from growth on maize wort agar containing 2% chalk. Temp. 30°. pH, 5.5.

Fig. 3. Oxygen uptake and CO_2 output, with 0.3 ml. 0.05 *M* calcium gluconate as substrate, of suspension of *Bact. suboxydans* from growth on maize wort agar containing 2% chalk. Temp. 30°. pH, 5.5.

time the evolution of carbon dioxide becomes equal in volume to the oxygen absorbed. The difference in volume between the total oxygen uptake and the carbon dioxide evolved represents the absorption of oxygen without any formation of carbon dioxide, that is, some simple oxidation process. In some cases this difference in volume becomes stabilized at about 330 $\mu\text{l.}$ which is approximately equivalent to one atom of oxygen for each molecule of glucose present. This excess of total oxygen uptake over carbon dioxide output is in all probability due to the formation of gluconic acid, which is oxidized as soon as produced giving an evolution of carbon dioxide equal to the oxygen absorbed.

In most experiments the difference in volume between oxygen uptake and evolution of carbon dioxide increases beyond 330 $\mu\text{l.}$ to about 450–470 $\mu\text{l.}$ This

additional excess of oxygen absorption may perhaps be accounted for by the partial oxidation of gluconic acid to 5-ketogluconic acid, $\text{CH}_2\text{OH} \cdot \text{CO}(\text{CHOH})_3\text{COOH}$, a reaction which has been observed for other acetic acid bacteria by several workers [Bernhauer & Schön, 1929; Hermann & Neuschul, 1931; Takahashi & Asai, 1931]. There are also the possibilities that 2-ketogluconic acid [Bernhauer & Görlich, 1935], 6-aldehydogluconic acid (*l*-glucuronic acid) [Takahashi & Asai, 1931; 1933] and saccharic acid may be formed. Another but less likely possibility is that there may be some oxidation process without evolution of carbon dioxide later on in the degradation of the glucose or gluconic acid molecule. However, the fact that 5-ketogluconic acid is a normal product of the glucose and gluconic acid metabolism of *Bact. suboxydans* in ordinary liquid culture points to this compound as being the most likely product in the experiments described above.

The view that gluconic acid is the first product of the action of the suspension of *Bact. suboxydans* grown on maize wort agar containing chalk is strengthened by the results obtained with 0.3 ml. of 0.05 *M* calcium gluconate in place of the equivalent 0.3 ml. of 0.1 *M* glucose as substrate in the Barcroft apparatus (Fig. 3).

Here the experimental results obtained are very similar to those obtained with glucose, with the significant exception that the difference between oxygen uptake and carbon dioxide output, that is, the absorption of oxygen unaccompanied by any evolution of carbon dioxide, rose only to about 120 $\mu\text{l.}$ after 5 hours. In the case of glucose, as stated above, the difference in most cases became stabilized at about 450–470 $\mu\text{l.}$, and this difference was usually attained in less than an hour. It will be noted that the two sets of figures for glucose and gluconic acid, i.e. 120 $\mu\text{l.}$ and 450–470 $\mu\text{l.}$, differ by 330–350 $\mu\text{l.}$, which is approximately equivalent to one atom of oxygen for 0.3 ml. substrate, that is, to the difference in oxygen content of the glucose and gluconic acid molecules.

Phosphatase activity of Bact. suboxydans.

Synthesis of phosphoric esters. All attempts to demonstrate the synthesis of hexosephosphates by the two types of suspension or their air-dried and acetone preparations gave negative results. The method employed was to make up flasks containing the following mixtures: 1 g. of air-dried or acetone bacteria or 1 ml. suspension, variable quantities of glucose ranging from 0.03 to 0.7 g., 0.07 ml. toluene and 2.5 ml. of 0.3 *M* Na_2HPO_4 for experiments on the alkaline side of neutrality or an equivalent quantity of KH_2PO_4 for experiments in acid conditions. The total volume was brought up to 10 ml. in each case and the flasks incubated at 30°. The amount of inorganic phosphate was determined [King, 1932] after 5, 10, 20, 30, 60 min. and afterwards at wider intervals up to 48 hours. No definite decrease in organic phosphate was observed in any experiment, though there were slight deviations on either side of the original phosphate value. These negative results do not necessarily mean that no hexosephosphates are synthesized, as they may be broken down immediately they are formed.

Hydrolysis of sodium hexosediphosphate. Simon [1930, p. 277] demonstrated the ability of *Bact. suboxydans* to hydrolyse magnesium hexosediphosphate to the extent of over 80 % in 48 hours.

In order to compare the hydrolytic powers of the two types of suspension and their acetone and dried preparations, the cultures from which they were derived were grown under the same conditions and on the same batch of maize wort agar, with the exception of the addition of chalk to the chalk-containing medium.

Mixtures of the following composition were made up:

0.5 g. acetone or air-dried preparation or 2 ml. suspension, 5 ml. buffer solution, 1 ml. of *M/7.5* sodium hexosediphosphate solution, the whole being made up to 10 ml. Potassium hydrogen phthalate buffer solutions of pH 4.5 were employed, preliminary experiments having shown that the optimum pH for the liberation of inorganic phosphate was between 4 and 5. The *M/7.5* sodium hexosediphosphate solution was prepared by dissolving 1.16 g. of anhydrous sodium sulphate in 30 ml. water, grinding with 2.44 g. of barium hexosediphosphate (B.D.H.) and centrifuging. Solutions containing bacteria and buffer, buffer and hexosephosphate were made up as controls. The flasks were placed in a thermostat at 30° and gently agitated. Samples were removed approximately every 24 hours and estimated for inorganic phosphate by King's method [1932].

Table I gives the results obtained in one experiment in which the suspension and acetone preparation of the two types were compared. Table II gives other experimental results obtained after varying periods of incubation at 30°. In this table the results within each experiment are strictly comparable. The results are expressed as mg. of inorganic phosphorus in each ml. of the mixture. The maximum possible value, for complete hydrolysis, is 0.83 mg./ml.

Table I. *Hydrolysis of sodium hexosediphosphate by Bact. suboxydans.*

A represents suspension or acetone preparation from maize wort agar containing 2% chalk.
B represents suspension or acetone preparation from maize wort agar without chalk.

Time hours	Suspension A dry weight 25 mg./ml.	Suspension B dry weight 23 mg./ml.	Acetone preparation A	Acetone preparation B
23	—	—	0.17	0.05
44	0.33	0.16	0.39	0.15
70	0.47	0.20	0.45	0.19
90	0.51	0.24	—	—
116	—	—	0.59	0.32

Table II. *Hydrolysis of sodium hexosediphosphate by Bact. suboxydans.*

Experiment	Culture grown on maize wort agar containing 2% chalk		Culture grown on maize wort agar with no chalk	
	Air-dried preparation	Acetone preparation	Air-dried preparation	Acetone preparation
No. 1 after 1 day	0.29	0.16	0.06	Trace
No. 1 after 3 days	0.43	0.53	0.10	0.10
No. 2 after 3 days	—	0.53	—	0.28
No. 3 after 1 day	0.44	—	0.09	—
No. 4 after 2 days	0.40	—	Nil	—

An examination of Table II shows that the results obtained were variable, but it is clear that there is a very definite difference between the two types in their power of forming inorganic phosphate from sodium hexosediphosphate. In one experiment (No. 4) and in one only, the air-dried preparation of the culture grown without chalk liberated no free inorganic phosphate, but in the other experiments the preparation derived from this medium showed some phosphatase activity but considerably smaller than that of the preparation derived from the maize wort agar containing chalk. It is therefore evident that the strong oxidative ability possessed by suspensions of *Bact. suboxydans* from cultures grown on maize wort agar containing 2% chalk is accompanied by considerably higher phosphatase activity than that possessed by the suspensions of restricted oxidative capacity derived from maize wort agar containing no chalk.

The effect of subculturing Bact. suboxydans of either type on to the characteristic medium of the other.

In order to determine whether the properties acquired by suspensions of *Bact. suboxydans* by growth on chalk maize wort agar were permanent or not, a culture which had been grown on the chalk medium for several generations was transferred to ordinary maize wort agar. Suspensions of the growth were made and examined for oxygen uptake and carbon dioxide output, while air-dried and acetone preparations of the suspensions were examined for phosphatase activity. All showed the characteristic properties of the type normally derived from maize wort agar. Similarly a culture which had been grown on ordinary maize wort agar for a long time immediately acquired the properties of the other variety when transferred to the chalk medium.

It is evident from this that the considerable modification induced in the enzyme system by the addition of chalk to the culture medium is a temporary variation and not a permanently acquired character.

SUMMARY.

1. *Bact. suboxydans*, an acetic acid organism characterized by its property of restricted oxidation in aerobic conditions, is found to possess a strongly oxidizing enzymic system when grown on maize wort agar containing 2% chalk.

2. Washed suspensions of the growth from maize wort agar without chalk are dirty white in colour and oxidize glucose to gluconic acid but no further at the optimum pH 5.5. There is no evolution of carbon dioxide. These suspensions have no action on gluconic acid.

3. Washed suspensions from maize wort agar containing chalk are pink and give a much larger and more rapid oxygen uptake with glucose as substrate than the other type of suspension, as well as a considerable evolution of carbon dioxide. Gluconic acid is first produced but is immediately oxidized with evolution of carbon dioxide equal to the oxygen absorbed in this reaction. Some keto- or aldehydo-gluconic acid, probably 5-ketogluconic acid, may also be formed. The action of this type of suspension on gluconic acid is very similar to that on glucose, and the difference in oxygen uptakes unaccompanied by evolution of carbon dioxide is accounted for by the difference of one atom of oxygen between the glucose and gluconic acid molecules.

4. No synthesis of hexosephosphates by suspensions or air-dried and acetone preparations of either type could be detected.

5. The suspensions and air-dried and acetone preparations of the cultures grown on the chalk-containing medium were found to possess a much greater capacity for hydrolysing sodium hexosediphosphate than the corresponding suspensions and preparations from the maize wort agar without chalk.

6. The modification in enzymic properties produced by the chalk is a temporary variation and not a permanently acquired character.

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